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Review

Cardiolipin binding in bacterial respiratory complexes: Structural and functional implications ☆

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ABSTRACT

The structural and functional integrity of biological membranes is vital to life. The interplay of lipids and membrane proteins is crucial for numerous fundamental processes ranging from respiration, photosynthesis, signal transduction, solute transport to motility. Evidence is accumulating that specific lipids play important roles in membrane proteins, but how specific lipids interact with and enable membrane proteins to achieve their full functionality remains unclear. X-ray structures of membrane proteins have revealed tight and specific binding of lipids. For instance, cardiolipin, an anionic phospholipid, has been found to be associated to a number of eukaryotic and prokaryotic respiratory complexes. Moreover, polar and septal accumulation of cardiolipin in a number of prokaryotes may ensure proper spatial segregation and/or activity of proteins. In this review, we describe current knowledge of the functions associated with cardiolipin binding to respiratory complexes in prokaryotes as a frame to discuss how specific lipid binding may tune their reactivity towards quinone and participate to supercomplex formation of both aerobic and anaerobic respiratory chains. This article is part of a Special Issue entitled: 17th European Bioenergetics Conference (EBEC 2012).

1. Introduction

Biological membranes govern a large number of cellular functions, and are actively involved in the function and spatial organization of membrane proteins. Understanding how the orchestrated and interdependent properties of lipids and proteins influence the functionality of biological membranes is a major topic of research in biology. It is widely recognized that the primary function of lipids is to define the permeability barrier of cells and organelles by forming a phospholipid bilayer. This bilayer serves as the matrix and support for a vast array of proteins involved in important cellular functions such as energy transduction, signal transduction, solute transport, DNA replication, protein targeting and trafficking, secretion, cell–cell recognition, motility, etc. More importantly, lipids do not play a static role in these processes but are known to govern folding, organization, and final structure of all membrane proteins. In addition, lipids directly influence and modulate the function of a large number of proteins that reversibly interact with the membrane surface. Hence, the importance of interactions between the lipid bilayer and the membrane proteins is increasingly recognized [1–3]. Despite a great deal of technical and methodological efforts [4–6], our understanding of lipid–protein interactions is limited by the number of membrane protein structures which account for less than 2% of the known high-resolution protein structures (328 unique structures to date in the Membrane Protein Data Bank [7]), although 20–30% of all genes in genomes encode for membrane proteins. While some phospholipids transiently interact with membrane proteins, others tightly bound to grooves and clefts on the protein surface or at subunit interfaces are retained during purification and often resolved in membrane protein structures. Hence, an increasing number of membrane protein structures revealed the presence of tightly bound endogenous lipids, some of them at specific sites thus paving the way to analyze the role of specific lipid interactions for the structure and function of membrane proteins [8]. A

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general trend in lipid binding is that the lipid headgroup is maintained through specific and mainly polar interactions while the position of fatty acyl or phytanyl chains nestled in grooves or clefts is maintained via a large number of non-polar interactions [9,10]. Noteworthy stability and functionality of membrane proteins are often controlled by binding of specific lipid species [2,11–14], the causal connection being in most cases not understood. An additional crucial parameter is the large diversity in lipid structures which enables a broad spectrum of chemical and physical properties for the membrane bilayer. Finally, the ability of lipids to form subdomains of unique protein and lipid composition provides a further control level to regulate and compartmentalize protein function within a membrane.

Among lipids, cardiolipin (CL) (1,3 diphosphatidyl-*sn*-glycerol) first isolated from bovine heart by M. Pangborn [15] has attracted much attention in the last decades (see for reviews, [16–21]), and is seen at present as a key component of energy-transducing membranes. CL is a non-bilayer anionic phospholipid exclusively found in the mitochondrial inner membrane and the chloroplast of eukaryotes, the cytoplasmic membrane of prokaryotes or the hydrogenosomes [17,22–25]. CL differs from all other lipids by its dimeric structure in which two stereochemically nonequivalent phosphatidyl moieties are linked by a central glycerol (Fig. 1). Although the presence of two phosphate groups may give rise to two negative charges, at neutral pH, CL contains a single charge because one proton gets trapped in a bicyclic resonance structure formed by the two phosphates and the central hydroxyl group. Moreover, due to the four acyl chains and the relatively small headgroup which has a high pK_a, CL can organize into domains which have the potential to serve as a proton sink particularly when in proximity to energy-transducing complexes that generate or consume ΔpH (transmembrane proton gradient) [26]. The conical shape of CL by favoring hexagonal phases and its clustering in the bilayer likely creates tension in biological membranes that is considered to be of primary importance for a number of processes such as membrane fusion, movements of proteins or even their spatial segregation [27,28].

One of the most prominent characters of CL is its ability to interact with a wide variety of membrane proteins, most of them being related to oxidative phosphorylation or photophosphorylation. In the late 80s and 90s, CL has been reported to modulate the catalytic activities and/or to provide stability to a number of interacting partners [29–39]. Since then, CL has been observed in the structure of the bacterial reaction centers [40,41], the bc_1 complex [42,43] or the cytochrome *c* oxidase [44,45]. In addition, CL has been seen to interact with the ADP/ATP carrier [46–48], the ATP synthase [39,49] or with the *Escherichia coli* succinate dehydrogenase [50,51], formate dehydrogenase [52] or nitrate reductase [53]. In most cases, CL binds preferentially at monomer interfaces of oligomeric assemblies and at subunit interfaces of multisubunit complexes, and acts as a flexible amphipathic linkage. Conserved and specific CL binding pockets were identified for the bacterial reaction centers, the cytochrome *c* oxidase or the bc_1 complexes [54–58] suggesting a specific requirement for this lipid at a particular site. A detailed survey analysis of the CL binding sites allowed to draw general principles for stabilization of this lipid, and the deduced characteristic binding pattern often consists of residues originating from different subunits [12,59].

Nevertheless, in regards to the description of conserved lipid-binding sites, it is not clear how CL contributes to full catalytic activity of energy-transducing complexes. The role of specific lipid–protein interactions has been studied in details in the mitochondrial cytochrome bc_1 complex in the seminal studies of C. Hunte's group [42]. This multi-subunit protein complex embedded in the inner mitochondrial membrane catalyzes the transfer of electrons from membrane-localized ubiquinol to water-soluble cytochrome *c*. This redox reaction is coupled to proton translocation across the membrane via a mechanism called protonmotive Q cycle formulated by P. Mitchell [60]. A wealth of biochemical, structural and biophysical studies has demonstrated that specific tightly bound phospholipids are essential for the assembly, stability and function of the bc_1 complex [35–37,61–63]. In particular, CL binding has been considered to be involved in proton uptake at the ubiquinone reduction site [42] and in allowing supercomplex formation with the cytochrome *c* oxidase [61]. Indeed, thanks to the use of a Δ*crd*1 mutant of yeast in which the final biosynthesis step of CL is absent [64,65], CL has been seen to play an active role in supramolecular organization of the mitochondrial respiratory chain through stabilization of supercomplexes [66–71] or in the oligomerization of the ATP synthase responsible for the cristae morphology [72]. Finally, both the altered level and the fatty acid chain composition of CL have severe impacts on mitochondrial functions and have been linked to a variety of human diseases [73–76].

In a number of systems described above, the tightly bound CL might be seen as a prosthetic group of the protein, the removal of which eventually affects stability and/or function. In this review, we attempt to provide an overview on current knowledge of the functions associated with CL binding to membrane-bound complexes associated with respiration in prokaryotes and to present some future challenges to understanding such interplay.

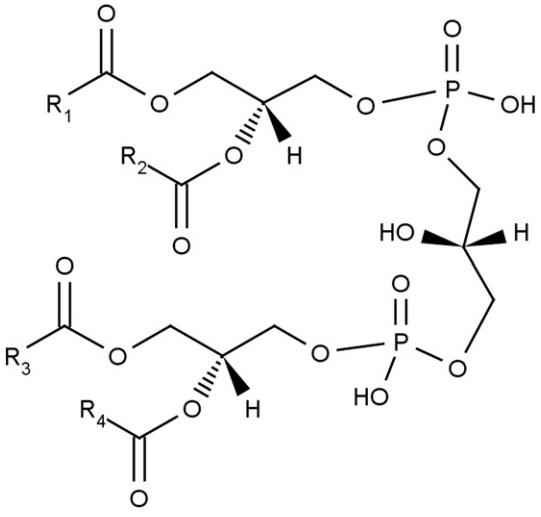

Fig. 1. Chemical structure of cardiolipin (1,3 diphosphatidyl-*sn*-glycerol). The two stereochemically nonequivalent phosphatidyl moieties are linked by a central glycerol. Four fatty acyl chains (herein referred to as R1 to R4) are linked via ester bond to the 1- and 2-hydroxyl groups of glycerol molecules of the polar headgroup (http://www.lipidmaps.org/) [242].

2. Cardiolipin in prokaryotes

Cardiolipin is found in most bacteria and archaea. First evidences for the existence of cardiolipin analogs in archaea came from the laboratory of A. Corcelli [22,77,78]. In archaea, CL is invariably composed of four identical phytanyl chains which contrasts with the diversity of the CL molecules in bacteria or in eukaryotes in terms of acid tail length, unsaturation or permutations of the chains. In addition a number of structural analogs of CL are found in bacteria such as *Streptococci* [79–82], *Clostridia* [83,84], *Listeria* [85,86], and *Geobacilli* [87], and it is conceivable that the era of lipidomics will reveal novel types. Finally, the presence of CL in hydrogenosomes, an organelle found in protists, was considered as an additional argument for the endosymbiotic hypothesis [23], according to which mitochondria were derived from an α-proteobacterium that lived inside an eukaryotic progenitor cell [88]. However, a striking difference between eukaryotes and prokaryotes is that mitochondria require a constant level of CL for basal activity, whereas prokaryotes have varying amounts of CL and may accumulate this lipid in specific situations depending on

their physiological state [89]. Although CL is a minor component during the exponential bacterial growth phase, its level increase in the stationary phase [90–94], in response to energy deprivation [95], osmotic stress [18,96–101], external pH [102] or even to the overproduction of fumarate reductase [103,104]. Another striking example is that spore membranes of *Bacillus subtilis* are CL enriched (25% of total phospholipids as compared to nearly 2% found in exponentially grown cells), CL being involved in an early step of spore germination [105].

Owing to its simple lipid composition, *E. coli* has been used as a model organism for understanding the individual roles of anionic lipids. *E .coli* membranes contain three major phospholipids: phosphatidylethanolamine (PE), a zwitterionic lipid which represents up to 70% of the total lipid content as well as two anionic ones, phosphatidylglycerol (PG) (20–25%) and CL (5–10%). The first evidence indicating that both anionic lipids, PG and CL were dispensable in *E. coli* came from the isolation of a *pgsA* mutant strain with only low levels of PG and CL [106]. It is worth mentioning that viability is only obtained through mutation of the *lpp* gene encoding a high copy outer membrane protein post-translationally modified by diacylglycerol derived from PG [107–109]. Indeed, in the *pgsA* mutant strain, the unmodified lipoprotein is covalently linked to peptidoglycan. This results in an anomalous anchoring of the inner membrane to peptidoglycan and disruption of cell envelope integrity [110]. Lipid analysis of the *pgsA*, *lpp* mutant strain revealed accumulated amounts of a phospholipid precursor, phosphatidic acid and *N*-acylphosphatidylethanolamine, two anionic lipids which may fulfill the structural and functional roles of PG and CL [111]. As expected, null mutants of the *cls* gene encoding for the major cardiolipin synthase in *E. coli* were found to have only a negligible growth defect despite an almost total lack of CL [112]. One explanation for the traces of CL in the *cls* mutant may be due to the existence of a second protein with CL synthase activity, YbhO while it remains unclear whether it contributes in vivo to CL cell content [113]. Moreover, a *cls ybhO* double mutant strain shows no growth defect [114]. Finally, it is well accepted that if the anionic lipids appear to be dispensable under laboratory conditions, PG and CL are required for optimal growth in natural environments.

During the last decade, our view on the organization of the bacterial cytoplasmic membrane has dramatically changed with the observation of distinct domains differing in their lipid and protein composition (see for review [115,116]). Accordingly, the cytoplasmic membrane has to be seen as an inhomogeneous and highly dynamic structure with defined membrane microenvironments [17,117,118]. Experimental evidence for the existence of lipid domains in bacteria came from the use of the hydrophobic fluorescent dye 10-*N*-nonyl acridine orange (NAO) displaying specific interaction with anionic lipids and in particular CL, and originally used to visualize mitochondria in eukaryotic cells [119,120]. A decade later, the first visualization of CL-enriched domains using NAO was reported in *E. coli* cells [121]. Most interestingly, these CL-enriched membrane domains were mostly observed at the cell poles and at the septal region of dividing cells. The same observations were made in *B. subtilis* [105,122] and *Pseudomonas putida* [123]. Furthermore, high levels of CL were detected in the engulfment and forespore membranes of *B. subtilis* [105] or in *E. coli* minicells derived from the cell poles [124]. Moreover, lipid microdomains similar to those of eukaryotic cells were reported in *B. subtilis* [125] providing an additional support to the mosaic character of the bacterial cytoplasmic membrane [117].

How can the existence of CL-enriched domains in the bacterial cytoplasmic membrane be explained? The intrinsic curvature of CL with a headgroup cross-sectional area much smaller than that of its lipid tails making it conical in shape has been considered as an essential parameter for CL microdomain formation [27,126]. Such a shape may be responsible for an asymmetry of distribution of CL within the bilayer with a specific enrichment at negatively curved regions of the inner leaflet of the bacterial membranes. Furthermore, it is considered that the bacterial cell wall constrains the cytoplasmic membrane to produce finite-sized clusters of high-curvature lipids such as CL which will spontaneously and stably localize to the cell poles and at the septal region in rod-shaped bacteria [27]. Such cell wall-mediated lipid microphase separation requires a critical amount of CL to produce domains which will form a lattice instead of a continuous CL-enriched domain as apparently visualized by NAO staining [127]. Additional support to this model came from a recent study showing that integrity of the cell wall peptidoglycan is required for the maintenance of the lipid domains in *B. subtilis* [128]. These lipid domains enriched in anionic phospholipids may in turn play an important role in compartmentalization of specific proteins in the membrane [129]. A prominent example is the amphitropic ATPase MinD involved in bacterial division-site selection which has been shown to oscillate between the cell poles and the septal region [130]. Membrane binding of MinD was shown to be mediated by an amphiphatic helix displaying affinity towards anionic lipids such as CL [131,132]. Polar and septal localization of MinD was thus related to binding to anionic phospholipid domains. Moreover, the membrane potential ($\Delta\psi$) has been reported to be involved in protein localization in several bacteria, in particular those involved in cell division such as MinD [133]. The use of valinomycin dissipating the $\Delta\psi$ results in a rapid loss of membrane binding and of polar oscillation of MinD. It remains to be addressed whether such effect may be in part due to a redistribution of the CL microdomains. Another example is the osmosensory transporter ProP in *E. coli* which has been shown to localize at the cell poles in a CL-dependent manner [134,135]. At present, systematic analysis of the relations between cell curvature and protein localization may be evaluated by the elegant method developed by the group of D.B. Weibel combining microtechnology and fluorescence microscopy [136].

On the other hand, anionic lipids have been shown to influence membrane protein topology through interactions between negatively charged phospholipids and positively charged aminoacids, working in opposition to the positive-inside rule at high concentration [137]. Anionic lipids also influence the functionality of several fundamental processes such as cell division [130], protein translocation [13,138–140] or respiration [53]. CL has been shown to tightly interact with the SecYEG protein complex stabilizing the dimeric form which in turn optimizes its functionality [141]. Interestingly, translocation of fully folded proteins through the Tat apparatus appears to be dependent on anionic lipids [142]. The signal recognition particle (SRP) receptor, FtsY, involved in cotranslational protein targeting in the inner membrane has also been shown to interact with anionic lipids, in particular CL [143,144]. Such interaction induces a conformational rearrangement of the FtsY protein through an allosteric mechanism resulting in activation of FtsY and of the SRP-FtsY complex [144]. CL has also been shown to be the most effective phospholipid to restore the activity of a number of purified respiratory complexes such as the NADH dehydrogenase [145,146], the lactate dehydrogenase [147], the succinate dehydrogenase [148,149], the cytochrome bo_3 ubiquinol oxidase [150] or the nitrate reductase A [53]. Not surprisingly, several X-ray structures have reported the presence of a tightly bound CL molecule to the formate dehydrogenase [52] and succinate dehydrogenase [50,51] in *E. coli*. More recently, a CL molecule has been considered to be present in the X-ray structure of the *E. coli* nitrate reductase A [53]. However, with the exception of *E. coli* nitrate reductase A, proofs that CL binding have a functional and/or structural role for these complexes are still lacking. It is worth to notice that formate dehydrogenase N and nitrate reductase A from *E. coli* represent the first two examples of anaerobic respiratory complexes interacting with CL. Each prokaryotic energy-transducing system reported to tightly bind a CL on the basis of the X-ray structure will be reviewed in detail in the following sections and be taken as examples of the interplay between CL and bacterial respiratory complexes.

3. The case of the respiratory nitrate reductase A from *E. coli*

Nitrate reductase A (NarGHI) is a cytoplasmic membrane-bound quinol-nitrate oxidoreductase that terminates the respiratory chain developed by *Escherichia coli* when grown anaerobically with nitrate as terminal electron acceptor [151,152]. Together with formate dehydrogenase N (FdnGHI), it generates a protonmotive force across the cytoplasmic membrane via a paradigmatic redox loop mechanism as proposed in the chemiosmotic theory of P. Mitchell [60]. Thus, NarGHI couples the oxidation of membrane ubiquinol or menaquinol at a periplasmically oriented Q-site (named Q_D) to the cytoplasmic reduction of nitrate and the concomitant net release of two protons in the periplasm. The X-ray crystal structure of NarGHI was solved at a resolution of 1.9 Å [153]. Crystal packing reveals that NarGHI forms a dimer (~500 kDa) with a two-fold symmetry axis approximately parallel to the membrane normal (Fig. 2A). Because of a large interacting area of more than 12.000 $Å^2$ between both monomers and the presence of bound endogenous phospholipids at the dimer interface, it was suggested that this organization is the physiological form of the enzyme. The heterotrimeric enzyme is composed of (i) a membrane-anchor subunit NarI containing two low-spin *b*-type hemes termed b_D and b_P to indicate their distal and proximal position to the catalytic site [154], (ii) an electron transfer subunit NarH that bears one Fe_3S_4 cluster and three Fe_4S_4 clusters [155,156], and (iii) a catalytic subunit NarG containing both a molybdenum cofactor and an additional Fe_4S_4 cluster [157–159]. These eight redox-active metal centers define a straightforward electron transfer chain from Q_D to the Mo atom at the active site [153]. The relatively large ~33 Å distance between redox centers from each monomer excludes any possible mechanistic role of the dimer.

Two partial phospholipids are also resolved in the NarGHI crystal structure (PDB ID: 1Q16). One is a putative phosphatidic acid located at the interface between the symmetrically related transmembrane subunits of the dimer (Fig. 2A). In addition, all the electron density maps associated with NarGHI or its mutants reveal a large region of extended electron density around both hemes b_D and b_P. A fraction of this density was originally attributed to a single PG molecule [153]. The rest could not be unambiguously modeled, and the presence of quinol molecules, detergents or lipid molecules was originally discussed. However, clear evidence for tight and specific binding of a CL molecule to NarGHI was recently provided [53]. Indeed, while the solubilization with increasing detergent concentrations of NarGHI-enriched inner membrane vesicles (IMVs) from *E. coli* leads to progressive loss of the NarGHI quinol oxidase activity without impacting the stability nor the dimeric state, analysis of the phospholipid content of these enzyme preparations by thin layer chromatography (TLC) indicated specific and tight binding of CL. Further, CL was shown to be the most effective phospholipid to restore functionality of a nearly-inactive detergent-solubilized enzyme complex reconstituted in proteoliposomes with various well-controlled lipid composition. These findings together with additional site-directed mutagenesis data led to reinterpret the extended electron density around both hemes which was then assigned to a CL [53]. In the model so-obtained, although the acyl chains of CL are almost exclusively in contact with the integral membrane subunit NarI, residues contributed by all three subunits stabilize its headgroup (Fig. 2B and C). Indeed, this crossroad is composed by two residues from the first α-helix of the catalytic NarG subunit (Arg6 and Tyr9), one residue from the NarH subunit (Arg218) and two residues from the NarI subunit (Tyr28 and Ser208). The acyl chains of CL are stabilized in a groove on the NarI subunit through numerous hydrophobic interactions (Leu21, Trp 25, Leu194, Phe195, Phe198, Ile206, Trp207, Met70 and Phe 63). Interestingly, the longest CL acyl chain that can be modeled in the electron density map with a length of 16 carbons runs along helix α2 and is in hydrophobic interaction with the above-mentioned Met70 and Phe63 as well as His66 in between them. The later interaction is of special interest as His66 is a ligand of heme b_D, suggesting a structural relationship between this heme and CL. Indeed, while two distinct heme b_D EPR signatures with g_z~3.20 and g_z~3.35 are observed in NarGHI-enriched IMVs likely due to two slightly different orientations of the imidazole rings of the histidine axial ligands, a decrease of the heme b_D heterogeneity was observed upon delipidation of the complex that was consistent with an interconversion of the two species [53]. Associated with spectral modification of heme b_D, significant modification of its redox properties was also observed (Arias-Cartin R, Grimaldi S, Guigliarelli B and Magalon A, unpublished results). Interestingly, a similar observation was made on the yeast bc_1 complex where both the spectral

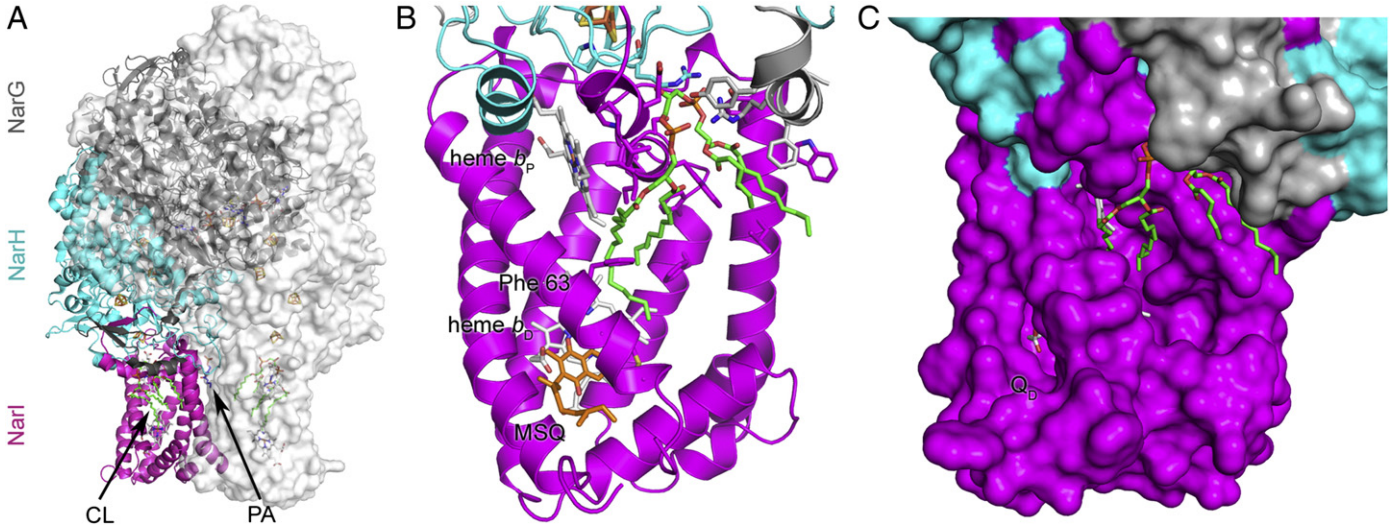

Fig. 2. Three-dimensional structure of the nitrate reductase A (NarGHI) from *E. coli* (PDB ID: 1Q16). Each subunit is represented as a cartoon and colored accordingly (NarI in purple, NarH in light blue and NarG in gray). Throughout the figures, the cofactors are represented as stick and their carbon atoms colored in white, nitrogen in blue and oxygen in red. Carbon atoms of cardiolipin are colored in green. (A) Overall structure of the NarGHI dimer showing the presence of tightly bound phospholipids. One monomer is shown in cartoon and in color as indicated above, the other one is shown in surface and colored in light gray. (B) Close-up view of the NarI subunit with the bound cardiolipin and the modeled menasemiquinone intermediate. This highlights the kink of the acyl chain that is in contact with the heme b_D and the participation of Phe63 in its stabilization. Residues making contacts with the cardiolipin molecule are shown in stick. (C) Same view as in (B) with the protein shown in surface. This highlights the engulfment of two acyl chains of the cardiolipin within a large hydrophobic groove of NarI and the proximity of the Q_D cavity at which quinol substrate binding occurs.

and redox properties of hemes b_L and b_H were dependent on the CL content of the enzyme. Such effect appears to be fully reversible upon CL addition [61].

A functional relationship between CL and NarGHI was found by considering that heme b_D and its His66 ligand are two essential components defining the NarGHI quinol oxidation site Q_D. Indeed, given the absence of an X-ray structure with bound quinone, it was previously shown that the EPR-detectable menasemiquinone and ubisemiquinone intermediates stabilized at the NarGHI quinol oxidation site Q_D (MSQ_D and USQ_D, respectively) cannot be formed in $NarGHI_{H66Y}$ mutants lacking the distal heme b_D [160–162]. Using the unpaired electron delocalized over the MSQ_D ring as a magnetic probe of its nuclear environment, high resolution pulsed EPR methods provided direct evidence for nitrogen-ligation to these radicals [162,163]. A multifrequency ^{14}N and ^{15}N HYSCORE study allowed its assignment to a Nδ imidazole nitrogen of a hydrogen-bonded histidine residue which was attributed to His66, the only His residue present in the NarGHI Q_D site. Further, the use of ^1H HYSCORE and ^2H ENDOR spectroscopies in combination with $^1H_2O/^2H_2O$ exchange experiments demonstrated a strongly asymmetric binding mode of MSQ_D via the formation of a single strong in-plane hydrogen bond to the His66 residue [164] (Fig. 2B). This atypical binding mode is currently proposed to strongly contribute to the unusually high redox stability of MSQ_D. Consistently, progressive delipidation of NarGHI-enriched IMVs was shown to drastically decrease the binding affinity to NarGHI of the substrate analog 2-heptyl-4-hydroxyquinoline *N*-oxide (HQNO) and to reduce the amount of EPR-detectable NarGHI-bound semiquinone without affecting its redox properties. Thus, one can hypothesize that the changes observed on the heme b_D g_z values upon enzyme delipidation may translate into slight variations of the orientation of the His66 imidazole ring [165,166] that directly impact the semiquinone hydrogen bonding. Overall, a tentative model that can reconcile all the data is the following: CL binding tunes the interaction with the quinol substrate by promoting a correct positioning of heme b_D and of its ligand His66 involved in the stabilization of the semiquinone intermediates [53].

Stabilization of the CL headgroup by electrostatic interactions with residues from all three subunits questions the role of CL in the heterotrimeric assembly of the complex. Folding and assembly of the NarGH complex has been shown to be a cytoplasmic event that occurs before anchoring to the membrane while complete maturation of NarI proceeds in a separate step that involves sequential insertion of the two *b*-type hemes [167–169]. Consequently, CL binding to NarGHI likely occurs after anchoring of NarGH to NarI, and can be considered per se as the final event of the biogenesis pathway.

While impacting strongly but reversibly on the activity of the enzyme, the absence of this CL molecule affects neither the enzyme stability nor its supramolecular organization [53]. This implies the possible coexistence of two states of the enzyme in the bacterial cell depending on CL binding. In *E. coli*, given that the expression level of cardiolipin synthase encoded by the *cls* gene is influenced by respiratory conditions and by the nature of the terminal electron acceptor [170], we envision that this CL-based respiratory complex activation may constitute an additional regulatory mechanism of bacterial respiratory chain activity depending on energy demand. Finally, the ability of a CL molecule to promote quinol substrate binding and stabilization to NarGHI may depend on the saturation of its acyl chains. Indeed, the particular positioning of the acyl chain in van der Waals interaction with the Cδ of His66 is likely due to an unsaturation of the fatty acid chain as can be deduced from the observed marked kink (Fig. 2B). Absence of the unsaturation or a *trans* conformation giving rise to a nearly straight conformation of the acyl chain may not provide the aforementioned contacts and the structural effect on the heme b_D.

One may question whether a similar situation can occur in other energy-transducing complexes known to display a CL dependence for their activity. One example is the bc_1 complex where the polar headgroup of a CL molecule is in proximity of the ubiquinone reduction site Q_i and heme b_H [42]. As mentioned above, it has been reported that delipidation is associated with significant spectral and electrochemical perturbations of both hemes, and in particular of heme b_H [61] but also with modifications of the protein backbone as measured by FTIR spectroscopy [63]. It remains open whether enzyme inactivation observed upon delipidation can partly be due to perturbation of quinone binding at the proximal Q_i site. In this context, studies on the *Rhodobacter sphaeroides* reaction center provide interesting observations. Indeed, a CL molecule is present in the crystal structure with the polar headgroup making contacts with all three subunits of the complex and situated at approximately 18 Å from both Q_A and Q_B, the primary and secondary acceptor quinones [40,41]. However, while the CL headgroup is positioned roughly equidistant from both quinones, CL has a substantial effect on the redox midpoint potential of Q_A [171,172]. CL appeared to lower the redox potential of Q_A in the isolated complex by approximately 40–50 mV, which brings the value more in accordance with the native membrane situation. It was ruled out that CL influences Q_A binding through a structural effect on the complex [173]. As such, anionic lipids and in particular CL regulate the bacterial reaction center functioning. Altogether, one has to consider that CL effect on the activity of energy-transducing complexes may, in some instances, be translated through their reactivity with quinone substrates.

4. The case of the formate dehydrogenase N from *E. coli*

Formate dehydrogenase N (FdnGHI) is a periplasmically oriented membrane-bound formate:quinone oxidoreductase that decomposes formate into carbon dioxide and protons. Formate oxidation is coupled to quinone reduction at a cytoplasmically oriented Q-site with the net release of two protons in the periplasm. As mentioned above, FdnGHI constitutes a respiratory chain together with nitrate reductase A (NarGHI) in *E. coli*. These two complexes are members of the widespread so-called complex iron-sulfur molybdoenzyme (CISM) superfamily of molybdo-pterin containing enzymes which play prominent bioenergetic roles in prokaryotes [169,174]. Recently, an evolutionary tree of key enzymes of the CISM superfamily reveals that several members, among which Fdn and Nar, were present already in the last universal common ancestor (LUCA) of prokaryotes [175].

The crystal structure of the formate dehydrogenase N was solved in its native form at a resolution of 1.6 Å and in complex with the menasemiquinone analog HQNO at a resolution of 2.8 Å [52]. Formate dehydrogenase N displays a heterotrimeric assembly composed of the three subunits FdnG (catalytic subunit), FdnH (single helix membrane spanning electron transfer subunit) and FdnI (integral membrane menaquinol binding subunit). Formate dehydrogenase N structure also revealed a physiological trimeric assembly (~480 kDa) that gives the enzyme an overall shape of a "mushroom" (Fig. 3A). Each monomer contains a ~90 Å long electron wire extending from the Mo atom of the molybdenum cofactor of FdnG to the hemes on FdnI. Electrons extracted at the Mo active site from the substrate are passed through six intermediate cofactors along the wire with first the Fe_4S_4 cluster on FdnG, then four Fe_4S_4 clusters on FdnH and hemes b_P and b_C on FdnI (b_P refers herein to the periplasmic side of the FdnGHI complex and b_C to the cytoplasmic side). Short edge-to-edge distances between the cofactors within a monomer prevent electron transfer between the trimeric assembly. In this architecture CL plays an important structural role as it is found not only at the interface of the trimer but also between subunits within each monomer (Fig. 3B). Indeed, the head group of CL is stabilized by Asn15 and Ser16 from FdnH and Thr39 from the FdnI subunit. The acyl chains of CL are at the crossroad of all this architecture with one chain pointing toward the heme b_C of FdnI on one monomer, another one in contact with hydrophobic residues of the membrane spanning helix of FdnH

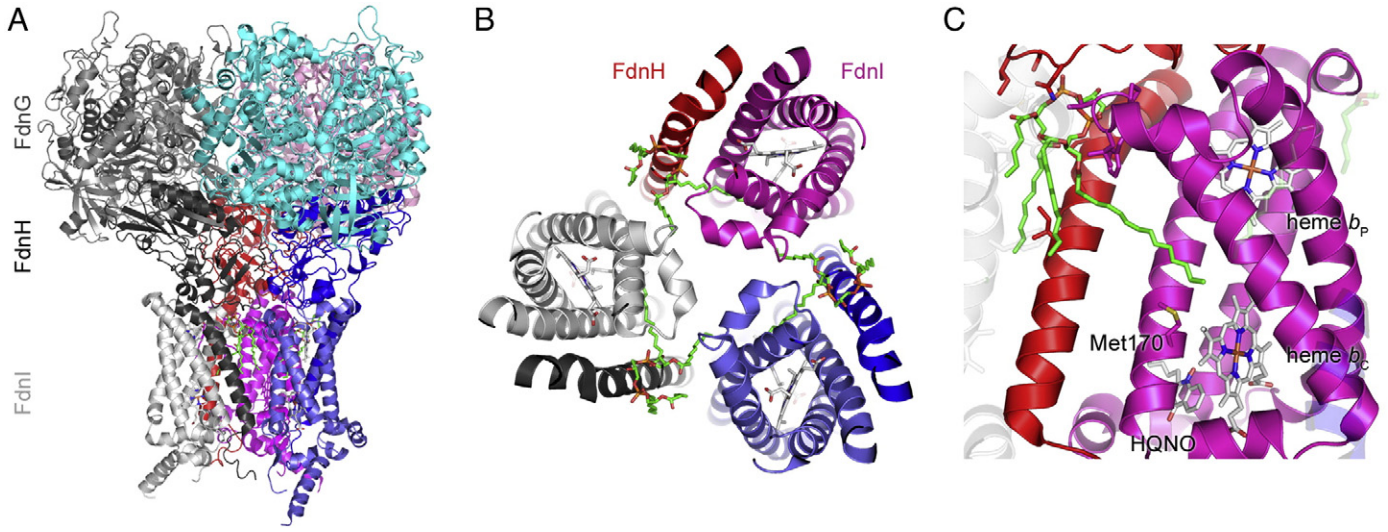

Fig. 3. Three-dimensional structure of the formate dehydrogenase N (FdnGHI) from *E. coli* (PDB ID: 1KQF). Throughout the figure, the FdnH subunit is darker than the integral membrane subunit FdnI. The cofactors are represented as stick and their carbon atoms colored in white, nitrogen in blue and oxygen in red. Carbon atoms of cardiolipin are colored in green. (A) Overall structure of the FdnGHI trimer. (B) Close-up view from the cytoplasm of the membrane anchored subunits and position of the cardiolipin molecules at the interface of the trimer. This highlights that two acyl chains point toward one monomer and the two others point toward an adjacent monomer. (C) Close-up view of the membrane anchored subunits of a monomer. Note the proximity of one acyl chain moiety of the CL molecule with heme b_C and the contact with Met170. The HQNO molecule (a menasemiquinone analog) is shown indicating the location of the quinone reduction site in the immediate proximity to heme b_C (PDB ID: 1KQG) and at the cytoplasmic side of the complex.

from the same monomer and the two remaining acyl chains in contact with hydrophobic residues of the FdnI subunit from an adjacent monomer (Phe26, Phe47, Met52 and Leu56) (Fig. 3C). In this context, CL seems essential for both trimer formation and inter-subunit interactions. Again one can wonder about the timing of the CL association to the FdnGHI complex. The periplasmic localization of the FdnGH catalytic dimer together with the presence of a twin-arginine signal peptide at the N-terminus of the unprocessed FdnG subunit [176] foresees cotranslocation of both metal center-containing subunits through the Tat machinery upon completion of the folding step [177]. Upon formation of the FdnGHI complex, positioning of the CL molecule on the FdnHI unit may trigger trimer formation.

As mentioned above, one acyl chain of CL is in contact with Met170 and points toward a vinyl group of the heme b_C of FdnI (Fig. 3C). This contact between one acyl chain of CL and heme b_C of FdnI is even stronger if one consider that "only" 15 carbon atoms of this acyl chain are stabilized in the crystal structure. Considering that the acyl chains of CL in *E. coli* are mainly composed of C16 or C18 carbon units but never of C15 ones [178,179], this means that a longer acyl chain will likely be in the immediate vicinity of heme b_C, although not stabilized enough to be visible in the electron density map. Therefore it is conceivable that CL plays a role in tuning the biophysical properties of heme b_C in the formate dehydrogenase N complex in a similar manner to NarGHI as reported previously [53]. The heme b_C was further shown to be in contact with HQNO in the crystal structure of the complex (PDB ID: 1kqg). Interestingly, the mode of interaction of the HQNO molecule resembles the one described for the endogenous semiquinone intermediates stabilized in the Q_D site of NarGHI since the *N*-oxide group of HQNO (analogous to the carbonyl oxygen O_1 of menaquinone) makes a hydrogen bond with the Nδ imidazole nitrogen of one heme ligand, His169 [52]. Thereof, a heme b_C ligand may be directly involved in quinone binding. Whether or not CL binding to FdnGHI influences some of its functional properties such as reactivity towards the quinone substrate through structural perturbations of heme b_C remains to be elucidated. Even though the binding site of CL is different in the nitrate reductase and formate dehydrogenase complexes, it is interesting to note that in both systems CL makes extensive contacts with different subunits in the complexes and, most of all, comes in contact with the heme that receives or donates electrons to the quinone substrate (heme b_D in NarI and heme b_C in FdhI, respectively). It is therefore conceivable that CL plays a similar functional role in both enzymes i.e. stabilizing the oligomeric state and modulating the reactivity towards quinone. This hypothetical model also opens the possibility to tune the whole redox loop system by modulating CL level and its degree of saturation in the membrane.

5. The case of the succinate dehydrogenase from *E. coli*

The succinate dehydrogenase is a multi-subunit enzyme that couples the oxidation of succinate with the reduction of quinone, a reaction involved in the tricarboxylic acid cycle. Notably, this complex is a member of the succinate:quinone oxidoreductase superfamily which shares several functional and structural characteristics and comprises the mitochondrial complex II and bacterial quinol:fumarate oxidoreductases (for review see [180]). *E. coli* SdhABCD has usually been used as a paradigm to study structure–function relationships of this group of proteins. In detail, this complex is anchored at the cytoplasmic side of the inner membrane and is assembled in three different domains: a FAD-binding protein domain (SdhA) responsible for the oxidation of succinate to fumarate, an iron-sulfur protein domain (SdhB) charged of the electron transfer through one Fe_2S_2, one Fe_4S_4 and one Fe_3S_4 until the hydrophobic domain assembled by two transmembrane polypeptides (SdhC and SdhD) and one heme *b* cofactor located near the cytoplasmic interface (b_P) [50,181]. The X-ray crystal structure of the succinate dehydrogenase from *E. coli* was solved at a resolution of 2.6 Å and shows a trimeric structure (~360 kDa) (Fig. 4A) [50,181]. Interestingly, the membrane subunit could be encoded as a single protein and/or diverge from the number of heme *b* cofactors among other succinate:quinone oxidoreductase members allowing the classification in five types [182,183]. A prominent example is the *Wolinella succinogenes* quinol:fumarate oxidoreductase homolog in which the hydrophobic domain is constituted by a single polypeptide (subunit C) with two cytochromes *b*, called proximal (b_P) and distal (b_D) [184]. These differences are known to significantly contribute to the location, number and reactivity on the quinone binding site(s), and as a consequence to the electrogenic character of the reaction [185,186].

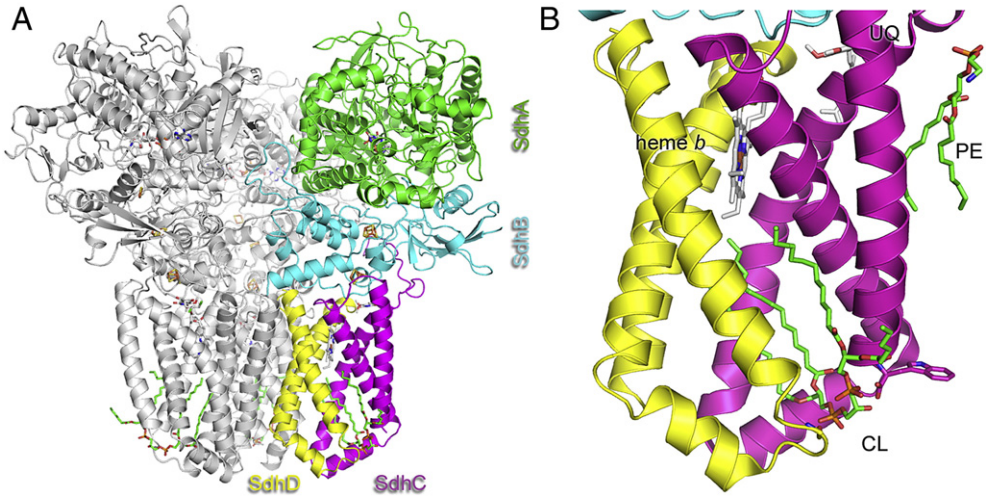

Fig. 4. Three-dimensional structure of the succinate dehydrogenase (SdhABCD) from *E. coli* (PDB ID: 1NEK). Each subunit is represented as a cartoon and colored accordingly (SdhA in green, SdhB in cyan, SdhC in magenta and SdhD in yellow). Throughout the figure, the cofactors are represented as stick and their carbon atoms colored in white, nitrogen in blue and oxygen in red. The same color representation is used for the ubiquinone substrate. Carbon atoms of cardiolipin and of phosphatidylethanolamine are colored in green. (A) Overall view of the trimer. (B) Close-up view of the membrane anchored subunits (SdhCD) with the bound CL molecule literally engulfed in the SdhC and SdhD interface. One of the acyl chains is in van der Waals contact with the heme at a distance below 3 Å while the polar headgroup is periplasmically oriented. Residues making contacts with the cardiolipin molecule are shown in stick. The PE molecule is also shown and likely forms part of the lipid annulus. Position of the UQ substrate is also shown at the interface with the cytoplasmic catalytic dimer.

The functional role of anionic lipids and in particular CL in succinate dehydrogenase complexes has been poorly studied, though a handful of elements have emerged from the X-ray structures and enzymatic studies. Early works have shown that succinate dehydrogenase activity could be restored in isolated *E. coli* membranes after incubation with mixtures of phospholipids and quinones, CL being the most effective [148]. In addition, CL stimulates the activity of a solubilized succinate dehydrogenase complex from *Mycobacterium phlei* [149]. Resolution of the X-ray structures of the *E. coli* SdhABCD complex revealed the presence of two well-ordered phospholipid molecules, one PE and one CL (Fig. 4B) [50,51]. In particular, CL is literally engulfed in the SdhC and SdhD interface and occupies the hydrophobic space above the heme b_P which accommodates an additional heme *b* in *W. succinogenes* quinol:fumarate oxidoreductase structure. The CL phosphatidyl groups point toward the periplasmic side of the membrane and are maintained by direct hydrogen bonds with residues originating from both SdhC (Trp129) and SdhD (Gly41 and Leu43) subunits. Also, a water molecule stabilized by residues Phe37 and Gly41 clearly plays a key role in the fixation of the CL headgroup. Furthermore, two acyl chains of 13 and 15 carbon atoms extend inside the hydrophobic cavity made within the SdhCD domain and are stabilized by several hydrophobic contacts with residues originating from both subunits. One of these acyl chains is in van der Waals contact with the heme with a distance below 3 Å. Noteworthy, this intimate lipid–protein association in *E. coli* SdhABCD and positioning of CL is rarely seen in other respiratory complexes, CL being most often found at monomer interfaces of oligomeric assemblies or at the surface of multisubunit complexes. However, as compared to the *E. coli* FdnGHI heterotrimer, the role of CL in the trimer formation of SdhABCD remains unclear owing to its particular positioning and the absence of contact of the acyl chains with neighboring monomers. Recently, Ruprecht and coworkers reported additional X-ray structures of the *E. coli* SdhABCD complex which do not show convincing electron densities for lipid molecules in the region where CL was first identified [187,188]. One reason could originate from slight modifications of the purification procedure, essentially exchanging the nonionic detergent Thesit by an amphiphilic neopentyl glycol detergent (DM, Anatrace) in downstream steps used for crystallization. As expected, the absence of CL left behind a considerable hydrophobic groove in these structures and an increased *B*-factor of the transmembrane subunits SdhCD. From these observations the authors claimed a stabilizing role of CL in these membrane-embedded proteins while being not essential for integrity of heme *b*. In light of these results, the function of CL in *E. coli* SdhABCD should be reconsidered since earlier studies have reported the beneficial effect of CL towards succinate dehydrogenase activity of the isolated complex [148]. Hence, CL could fulfill another function than previously noted. It is tempting to speculate that heme *b* positioning and/or redox potential is modulated by the close proximity of the CL acyl chain (~3 Å). However, if such an interaction exits, it could only be relevant to a subset of succinate:quinone oxidoreductase complexes because the same positioning of this lipid in other homologs might interfere with the insertion of a b_D cofactor or the integrity of a Q_D site. Furthermore, in this perspective CL would have a reduced effect in *E. coli* succinate dehydrogenase activity since the role of the heme cofactor still remains enigmatic. In fact, *E. coli* SdhC and/or SdhD mutants lacking the heme *b* retain nearly half of the enzyme turnover and did not produce an excess of reactive oxygen species [189]. Similar results were observed upon mutation of the axial ligands of the heme in *Saccharomyces cerevisiae* complex II showing no effect on enzyme assembly and activity [190,191]. In any case, establishing a function of CL in *E. coli* SdhABCD would be important to reconcile the available data.

6. Role of cardiolipin in supramolecular organization of the respiratory chain in bacteria

In regard to the occurrence of CL within the X-ray structure of several bacterial respiratory complexes, one may question the implication of CL in the supramolecular assembly of the bacterial respiratory chain in a similar manner to the situation encountered in the inner membrane of mitochondria. Indeed, in eukaryotes, the enzymes involved in the oxidative phosphorylation are assembled in highly-ordered supercomplexes within the mitochondrial inner membrane [192–195]. The most prominent examples are those comprising complexes I–III–IV or the dimeric/oligomeric forms of the ATP synthase with representatives from fungi, plants and mammals. These observations have profoundly changed the view on the organization of the respiratory chain and subsequently led to challenge the previously accepted model, the random diffusion model, stating that the complexes are randomly distributed in the lipid bilayer but are

functionally connected by diffusible electron carriers such as quinones and cytochrome *c* (see for instance, [196]). The actual view is that the components of the respiratory chain are in equilibrium between isolated state and supercomplexes, depending on metabolic conditions of the cell [196–198]. In this context, a change in the respiratory state of the mitochondria induces changes in CL distribution [199]. The role of CL in cristae morphology was recently supported by the observation that CL is responsible for the supramolecular assembly of ATP synthase [72]. In a more general sense, CL is specifically required for supercomplex association and stabilization [61,66,69,71,200–202]. Even more, distortion of the lipid bilayer induced by peroxidation which targets unsaturation of the fatty acid chains of CL results in disruption of the supercomplexes [203–205].

Respiratory chain supercomplexes have also been detected in bacteria and archaea but one has to keep in mind that a specificity of prokaryotes is their extreme respiratory flexibility. In addition to oxidative phosphorylation, most prokaryotes have the ability to use alternative, inducible respiratory pathways using terminal electron acceptors other than molecular oxygen. *E. coli*, a facultative anaerobe, apart the aerobic respiratory chain composed of type I and II NADH dehydrogenases, succinate dehydrogenase and three quinol oxidases (*bo*$_3$, *bd*-I and *bd*-II) [206,207], can express a large array of dehydrogenases and reductases depending on the environmental conditions [206,208]. Most of the reported supercomplexes in prokaryotes involved complexes participating in the aerobic respiratory chain as illustrated in *Sulfolobus* [209,210], *Paracoccus* [211,212], *Bacillus* [213,214], *Corynebacterium* [215], *Acidithiobacillus* [216], *E. coli* [217] and *Aquifex* [218,219]. Furthermore, fluorescence microscopy allowed to observe that ATP synthase and succinate dehydrogenase are heterogeneously distributed in mobile patches in the cytoplasmic membrane of *B. subtilis* [220]. Identical observation was made on the cytochrome *bd*-I in *E. coli* [221]. Consequently, compartmentalization of processes related to respiration in the bacterial cytoplasmic membrane has been hypothesized leading to the notion of respirazones, i.e. specialized compartments dedicated to respiration [222].

Surprisingly, supramolecular organization of complexes involved in anaerobic respiration has not yet been reported. Since binding of specific lipids is a determinant of supercomplex organization, CL binding to a number of anaerobic respiratory complexes in bacteria, such as formate dehydrogenase N (FdnGHI) [52] and nitrate reductase A (NarGHI) [53] may have additional roles. These two complexes constitute a major alternative respiratory pathway in several prokaryotes, the nitrate respiratory pathway, induced by anaerobiosis and the presence of nitrate [151]. As mentioned before, within the heterotrimeric structure of FdnGHI, each CL tightly interacts with FdnHI subunits of one heterotrimer and with the FdnI subunit of the neighboring heterotrimer. While one of the acyl chains fills in a tunnel leading to the heme b_c in proximity of the quinone-binding site, others are involved in interactions with neighboring subunits suggesting the important role of CL in the trimer formation [52]. The crystal packing of NarGHI also revealed a dimer with a two-fold symmetry axis approximately parallel to the membrane normal [153]. Although the membrane integral subunit NarI does not seem to play a prominent role in this dimerization (it contributes to less than 10% of the surface contact), the role of lipids in the dimerization is not excluded. Indeed a phospholipid molecule originally attributed to a phosphatidic acid is present at the interface of the dimer, and NarI from one heterotrimer together with the swapped domain of NarH from the other heterotrimer stabilizes this lipid. It is also possible that this phospholipid molecule is in fact an additional molecule of CL as it is suggested by detergent-solubilization of NarGHI [53]. Using a large excess of detergent was not sufficient to resolve the dimeric structure of the NarGHI complex nor the release of a CL molecule found in stoichiometric amount in the nearly inactive detergent-solubilized complex. The role of CL would therefore extend to the dimerization of the NarGHI heterotrimer in the same way as in complexes III, IV and V [39,42–45] or the ADP/ATP carrier [48]. For instance, the dimeric state of the bovine complex IV is strongly stabilized by CL making extensive contacts with each monomer [45]. Interestingly, complex IV associates in the form of monomers within the respiratory supercomplex $I_1III_2IV_1$ from bovine heart mitochondria [223]. The complex IV face interacting with complex III is the one involved in the dimerization and making contacts with CL. Consequently, it has been proposed that the contact interface of complex IV involved both in the dimer and in the supercomplex might participate in the equilibrium between isolated and highly-ordered complexes. In a similar manner, reorganization of the oligomeric structures of both FdnGHI and NarGHI might allow formation of a supercomplex where the contact interface of FdnGHI involved in CL binding within the heterotrimer interacts with the NarGHI complex. Such organization may position the quinone binding site of FdnGHI (as seen from HQNO binding) in proximity to the quinol-binding site cavity Q_D within NarGHI. Such arrangement resulting in short diffusion distances for quinone molecules may increase the electron transfer efficiency from FdnGHI to NarGHI as proposed in the solid-state model. In support of this hypothetic model is the reported co-purification of FdnGHI and NarGHI complexes over a wide-range of conditions [224,225] which may be suggestive of their physical association.

7. Conclusions and future prospects

The importance of CL in cell function has received much attention during the last decades. As discussed in this review, we focused on the functions associated with this peculiar anionic phospholipid in membrane proteins associated with respiratory function in prokaryotes. *E. coli*, a facultative anaerobe, has the ability to synthesize several respiratory chains with a large variability in composition depending on the availability of oxygen and other electron acceptors. To date 15 primary dehydrogenases and 11 terminal reductases or oxidases have been identified in *E. coli* [206,226]. These complexes are further linked by different types of quinones, ubiquinone, menaquinone and demethylmenaquinone with varying ratios depending on the growth conditions [206,227–229]. The resulting respiratory flexibility serves to optimize metabolism according to the nature of electron donors and acceptors. In this context, the existence of respiratory chain supercomplexes in prokaryotes adds a further level of regulation and complexity which need to be further studied kinetically and structurally. In particular, given the diversity of electron acceptors other than oxygen in prokaryotes, it is likely that a number of alternative supercomplexes will be discovered in the future.

The unique lipid composition of a specific cell membrane could also affect the function of a protein. The most prominent example is CL, found in the inner membrane of mitochondria in eukaryotes, which controls the activity of a number of respiratory complexes as well as the structural organization of the inner membrane. Recent evidence of localization of CL to the poles and septal regions of bacterial cells suggests that polar targeting of some proteins may depend on this membrane heterogeneity [134]. In this context, fluorescence microscopy has been an excellent tool to follow cellular distribution of lipids and respiratory complexes and their dynamics [121,221]. As reported in this review, a number of respiratory complexes require CL binding for their activity. These complexes may further require to be localized to these CL-enriched domains for optimal activity. Such spatial segregation of proteins may be in turn at the origin of the formation of supercomplexes. Interestingly, the group of Dieter Jahn has recently reported the existence of several alternative electron transport chains consisting of the penultimate key enzyme in heme biosynthesis, HemG in *E. coli*, and of quinol-oxidizing complexes such as quinol oxidases, fumarate reductase and nitrate reductase A [230]. Orchestration of physiological processes such as respiration and related processes in microdomains may thus be a more widespread feature of membranes than previously appreciated. Overall,

varying the amount of CL in the prokaryotic cell can temporally and spatially provide an elegant, mechanistically simple way of turning on or off the activity of respiratory complexes and to influence the higher-organization of the respiratory chain depending on the energy demand.

In this context, one may thus question what is then the situation in the absence of CL? To date, the *cls* mutant strain contains traces of CL while the *cls ybhO* double mutant strain is still viable indicating that a strong reduction of CL level does not have a severe effect on growth ability of *E. coli* cells under aerobic conditions [114]. Disruption of all gene encoding proteins with CL synthase activity would be necessary but it may well be that PG partially compensates the reduction in CL level as reported in mitochondria [64]. For instance, using proteoliposomes with varying lipid composition, PG was shown to functionally substitute CL for NarGHI activity albeit at a lower level [53]. However, again, the *lpp pgsA* mutant strain lacking both PG and CL is viable under aerobic conditions probably due to the presence of two other anionic phospholipids [111]. It remains open whether the combined defect in PG and CL has an influence on anaerobic growth and on the functionality of respiratory complexes. In this review, we discuss many potential implications for CL in bacterial respiration including in particular, a spatiotemporal dimension. In this context, we consider that new experimental knowledge is needed at single cell level taking advantage of the recent achievements in fluorescence microscopy.

Membrane proteins are surrounded by lipids that are often partly resolved in X-ray crystal structures providing only a part of the actual environment of proteins. In addition, with the exception of NarGHI, FdnGHI and SdhABCD from *E. coli*, the X-ray structure of a number of respiratory complexes in prokaryotes has not revealed the presence of bound lipids [184,231–236] reinforcing the difficulties of studying lipid–protein interactions. Recent advances in electron crystallography allow now to obtain high resolution structure of both the protein and its surrounding membrane [237,238]. Provided that automation and high throughput methods are developed, such approach can be seen as a very promising and powerful technique. Considering that lipid composition of biological membranes is not uniform temporally and spatially, and that changes in lipid environment can strongly influence membrane protein organization and/or function, direct evaluation of the lipidome is required and can now beneficiate from recent advances in mass spectrometry [239]. While relatively non-specific lipid–protein interactions occur in the lipid annulus, the situation is clearly different for buried lipids such as CL mostly found at interfaces of oligomeric assemblies and at subunit interfaces of multisubunit complexes. In this context, recent developments of Förster resonance energy transfer (FRET) technology [240] or time-resolved fluorescence microscopy [241] can benefit to studies of lipid–protein interactions by giving access to temporal and spatial dynamics in living cells. Altogether, we consider that the outcomes of the abovementioned approaches may disclose unprecedented relations between lipids and membrane proteins.

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